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Abstract: Low density lipoprotein cholesterol (LDL) and apolipoprotein B-100 (1 to 15 g/mL) had no significant influence on the inositol-1,4,5-trisphosphate (InsPa) formation in vascular smooth muscle cells and fibroblasts. Low density lipoprotein cholesterol (15 g/mL) induced an elevation of intracellular Ca²⁺ from 85 to approximately 210 nmol/L in vascular smooth muscle cells from rat aorta in the absence or in the presence of 15 g/mL monoclonal antibodies against the classical low density lipoprotein receptor or in the presence of apolipoprotein B-100. Moreover, in both human cultured fibroblasts from normocholesterolemic individuals and from patients with familial hypercholesterolemia homozygote class 1, LDL induced a dose-dependent rise of free intracellular calcium and a biphasic change of intracellular pH. Since homozygote class 1 fibroblasts are classical LDL receptor negative, and as antibodies against this receptor, as well as apolipoprotein B-100, did not attenuate the LDL-induced elevation of cytosolic calcium, we conclude that LDL might modify vascular activity via the observed intracellular changes without involving the classical low density lipoprotein receptor. *Am J Hypertens* 1991;4:274-279

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Generation of Intracellular Signals by Low Density Lipoprotein Is Independent of the Classical LDL Receptor

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Low density lipoprotein cholesterol (LDL) and apolipoprotein B-100 (1 to 15 $\mu\text{g/mL}$) had no significant influence on the inositol-1,4,5-trisphosphate (InsP_3) formation in vascular smooth muscle cells and fibroblasts. Low density lipoprotein cholesterol (15 $\mu\text{g/mL}$) induced an elevation of intracellular Ca^{2+} from 85 to approximately 210 nmol/L in vascular smooth muscle cells from rat aorta in the absence or in the presence of 15 $\mu\text{g/mL}$ monoclonal antibodies against the classical low density lipoprotein receptor or in the presence of apolipoprotein B-100. Moreover, in both human cultured fibroblasts from normocholesterolemic individuals and from patients with familial hypercholesterolemia homozygote class 1, LDL induced a dose-dependent rise of

free intracellular calcium and a biphasic change of intracellular pH. Since homozygote class 1 fibroblasts are classical LDL receptor negative, and as antibodies against this receptor, as well as apolipoprotein B-100, did not attenuate the LDL-induced elevation of cytosolic calcium, we conclude that LDL might modify vascular activity via the observed intracellular changes without involving the classical low density lipoprotein receptor. *Am J Hypertens* 1991;4:274-279

KEY WORDS: Low density lipoprotein, vascular muscle cells, fibroblasts, intracellular Ca^{2+} and pH, phosphatidylinositol turnover.

Recently, a correlation between blood pressure and serum cholesterol levels has been described, suggesting that those patients with higher blood pressure values tend to have higher cholesterol levels.¹ Low density lipoprotein, the major cholesterol-carrying lipoprotein of human plasma, delivers cholesterol to cells by binding to the classical LDL receptors. The cholesterol is then internalized by endocytosis. These receptors recognize the apo-

lipoprotein B-100 hydrophobic polypeptide.² Low density lipoprotein is considered to be the main atherogenic class of lipoproteins, and contains 60 to 70% of the total serum cholesterol. Epidemiological and pharmacological studies indicate that the lowering of LDL-cholesterol is accompanied by a decrease in the risk of hypertension.^{3,4} These results suggest that increased LDL-cholesterol levels might modify vascular reactivity in humans with hypercholesterolemia. In addition, vascular smooth muscle cells (VSMC) play a key role in the pathophysiology of both hypertension and atherosclerosis.^{5,6} In order to elucidate cellular mechanisms by which LDL could be involved in the development of hypertension, we demonstrated very recently in cultured VSMC that LDL can induce an elevation of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) and a biphasic change of intracellular pH (pH_i) via the Na^+/H^+ exchanger. In addition, LDL induced vasoconstriction of rat thoracic aortic rings.⁷

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In the present report, we examined whether these intracellular changes could be mediated by the classical LDL receptor via stimulation of the phosphatidylinositol (Ptlns) turnover, and thus leading to a release of cytosolic calcium. For this purpose, we investigated the effect of LDL in VSMC in the presence of monoclonal LDL receptor antibodies (IgGC₇)⁸ and apolipoprotein B-100 on the Ptlns turnover as well as on $[Ca^{2+}]_i$ and pH_i . Moreover, we studied the effect of LDL on $[Ca^{2+}]_i$ and pH_i in LDL receptor negative cultured fibroblasts from patients with familial hypercholesterolemia (FH) homozygote class 1 mutations. Genes from these mutations cannot produce any LDL receptor protein.²

MATERIALS AND METHODS

Materials Fura 2/pentaacetoxymethyl ester (fura 2/AM) and [2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein]-pentaacetoxymethyl ester (BCECF/AM) were obtained from Calbiochem (Zürich, Switzerland). Dulbecco's modified Eagles medium (DMEM), Ham's F-10 and Dulbecco's phosphate-buffered saline (PBS) were obtained from Amimed (Zürich, Switzerland). Ethyleneglycol-bis-(2-aminoethyl) tetraacetic acid (EGTA), 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES) Tris(hydroxymethyl)aminomethane (Tris-base), and other chemicals were obtained from Sigma Chemical (Zürich, Switzerland). Monoclonal low density lipoprotein antibodies as well as myo-[2-³H]inositol, and standard [³H]-Inositolpolyphosphates were obtained from Amersham International, Amersham, Buckinghamshire, England. Normal and Class 1 FH homozygote fibroblasts were obtained from The Human Genetic Mutant Cell Repository Institute for Medical Research, USA.

LDL Isolation Low density lipoprotein (density, 1.019 to 1.063 g/mL) was isolated from the plasma of 5 individual normocholesterolemic subjects (serum cholesterol < 6.2 mmol/L) by ultracentrifugation according to Redgrave et al.⁹ The LDL fraction was dialyzed against 0.15 mol/L NaCl/1 mmol/L EDTA, pH 7.4, and used within 3 weeks. Oxidation of LDL was prevented by adding 5 μ mol/L butylated hydroxytoluene (BHT) throughout. The LDL was stored at 4°C and used within 3 weeks; no changes in activity were observed during this period of time. Determination of protein was performed by Lowry's method.¹⁰

Culture of Vascular Smooth Muscle Cells Vascular smooth muscle cells were isolated from rat aorta (female, Wistar-Kyoto strain, 6 to 8 weeks old) and recultured several times in accordance with the method of Ross.¹¹ The cells were allowed to grow for 4 to 5 days in 5% CO₂, 95% air at 37°C. The culture medium was Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% fetal calf serum.

Culture of Fibroblasts Normal and FH homozygote fibroblasts were obtained from the Human Genetic Mutant Cell Repository Institute for Medical Research and recultured several times after detachment of the confluent cells with Puck's Saline A physiological solution containing 0.04% trypsin/0.02% EDTA buffer. The cells were allowed to grow as described for the VSMC.

Measurement of Inositolphosphates Cells were seeded in Petri dishes (60 mm diameter) and grew in the presence of myo-[2-³H]inositol (4 μ Ci/mL) for 3 days. During this time the cells reached confluence. They were washed three times with HEPES buffer, followed by a 2 h incubation in DMEM without FCS. After 2 other washes with buffer, cells were incubated for 20 min in DMEM containing 20 mmol/L LiCl in order to inhibit inositol-1 monophosphatase activity. Then cells were stimulated with LDL for various periods of time. The reactions were terminated by the addition of icecold 8% trichloroacetic acid (TCA). Samples were kept on ice for 30 min before removing TCA by extracting the samples with diethyl ether.¹² Radiolabeled inositol phosphates were analyzed and quantified by standardized anion exchange HPLC.¹³ The 1.25 mL fractions were transferred to scintillation vials and radioactivity was determined in the liquid scintillation counter. Peak identification of inositol-1-monophosphate, inositol-1,4-bisphosphate, and inositol-1,4,5-trisphosphate, were performed by using standard radiolabeled [³H]inositol phosphates. Cells were incubated with 1, 7, and 15 μ g/mL LDL for different time periods. Positive control experiments were performed by stimulation of the cells with 100 nmol/L endothelin for different time periods.

Measurement of Free Intracellular Ca²⁺ [Ca^{2+}]_i Confluent cells were detached with 0.04% trypsin/0.02% EGTA/Puck's Saline A physiological solution after 5 to 10 min at 37°C. Then cells were incubated with 2 μ mol/L fura-2 pentaacetoxymethyl ester at 37°C for 20 min in HEPES-buffer (20 mmol/L HEPES, 16 mmol/L glucose, 130 mmol/L NaCl, 1mmol/L MgSO₄ · 7 H₂O, 0.5 mmol/L CaCl₂, Tris-base, pH, 7.4) supplemented with 1% bovine serum albumin (BSA). After loading, cells were washed and suspended in HEPES buffer (approximately 2 × 10⁶ cells/mL). The Ca²⁺-fura-2 fluorescence was measured at 37°C while stirring in a SLM-Aminco SPF-500 (Urbana, IL), spectrofluorometer (excitation wavelengths: 340 and 380 nm; emission: 505 nm). Fluorescence was corrected for cellular autofluorescence. Fluorescence signals were calibrated using 0.5% Triton X-100 for measurement of maximum fluorescence followed by the addition of 2 mmol/L MnCl₂ for minimum fluorescence according to Grynkiewicz et al.¹⁴

Measurement of pH_i These measurements were performed using the method of Berk et al¹⁵ with the fluorescence pH indicator BCECF. Cells were loaded as described for the fura-2 loading method in HEPES buffer with 2 $\mu\text{mol/L}$ BCECF-pentaacetoxymethyl ester for 20 min at 37°C. For the fluorescence measurements the following wavelengths were set: excitation wavelengths: 492 and 438 nm; emission wavelength 525 nm. The calibration curve was performed by permeabilizing the cells with 30 $\mu\text{mol/L}$ digitonin as previously described.

RESULTS

LDL or Apolipoprotein B-100 Has No Effects on Inositolphosphate Formation In order to investigate whether LDL can stimulate the phosphatidylinositol (Ptlins) turnover, the formation of radiolabeled inositol-1 monophosphate (InsP_1), inositol-1,4-bisphosphate (InsP_2) and inositol-1,4,5-trisphosphate (InsP_3) in VSMC was determined by using standardized anion HPLC. Table 1 shows the time-dependency of the myo-[³H]inositol labeled InsP_1 , InsP_2 , and InsP_3 formation in VSMC resulting from endothelin and LDL stimulation. No significant synthesis of InsP_1 , InsP_2 , and InsP_3 could be observed after stimulation periods of 20 sec, 1, 3, 5, 10, and 20 min with 15 $\mu\text{g/mL}$ LDL or apolipoprotein B-100 compared to the unstimulated cells. Similar results were obtained with 3 and 7 $\mu\text{g/mL}$ LDL or apolipoprotein B-100. Endothelin induced a strong time-dependent formation of the inositol polyphosphate with a maximum at approximately 10 min. In comparison with unstimulated cells or cells in the presence of 15 $\mu\text{g/mL}$ LDL or apolipoprotein B-100, endothelin caused an approximately 6-, 13-, and 4-fold higher formation of InsP_1 , InsP_2 , and InsP_3 in VSMC, respectively.

Apolipoprotein B-100 or Monoclonal Antibodies Against the LDL Receptor (IgGC₇) Cannot Influence the LDL Induced Elevation of $[\text{Ca}^{2+}]_i$ Apolipoprotein B-100 (3,7,15 $\mu\text{g/mL}$) had no effects on $[\text{Ca}^{2+}]_i$. Preincubation of VSMC for 30 min with 15 $\mu\text{g/mL}$ apolipoprotein B-100 had no effect on the LDL induced release of $[\text{Ca}^{2+}]_i$. The maximal LDL induced elevation of $[\text{Ca}^{2+}]_i$, occurring at 20 sec, was 212 ± 19 in the absence and 208 ± 21 nmol/L in the presence of 15 $\mu\text{g/mL}$ apolipoprotein B-100 (mean \pm SD, $n = 4$, basal value 70 ± 9 nmol/L $n = 8$). Addition of various concentrations of IgGC₇ to VSMC for 30 min failed to influence $[\text{Ca}^{2+}]_i$. Preincubation of VSMC for 30 min with 1,7,35, and 70 $\mu\text{g/mL}$ IgGC₇ at 37°C, followed by a 15 $\mu\text{g/mL}$ LDL stimulation did not abolish or reduce the LDL induced elevation of $[\text{Ca}^{2+}]_i$.

LDL Elevates $[\text{Ca}^{2+}]_i$ in Normal and Receptor Negative Fibroblasts Figure 1 shows the concentration-response curve for the maximal LDL induced rise of $[\text{Ca}^{2+}]_i$ at 15 sec in the presence of extracellular Ca^{2+} . Low density lipoprotein concentrations ranging from 1 to 7 $\mu\text{g/mL}$ caused a dose-dependent increase of $[\text{Ca}^{2+}]_i$. Low density lipoprotein concentrations higher than 7 $\mu\text{g/mL}$ caused no further significant rise of $[\text{Ca}^{2+}]_i$. Similar results were obtained in normal fibroblasts in both the presence and absence of extracellular Ca^{2+} . As demonstrated in Figure 2a, 15 $\mu\text{g/mL}$ LDL induced, in the presence of extracellular Ca^{2+} , a rapid rise in $[\text{Ca}^{2+}]_i$ (basal value = 73 ± 8 nmol/L, mean \pm SD, $n = 12$) with a maximum at 15 sec. The rise of LDL induced stimulation of $[\text{Ca}^{2+}]_i$ declined after 15 sec and reached the resting level within 1.0 min. Low density lipoprotein also stimulated a rapid rise in $[\text{Ca}^{2+}]_i$ in the Ca^{2+} -free buffer (Figure 2b). This rise, however, was less pronounced than in the presence of extracellular Ca^{2+} .

TABLE 1. EFFECT OF LDL AND ENDOTHELIN ON INOSITOLPOLYPHOSPHATES FORMATION IN VSMC (MEAN*)

Time (min)	Stimulated with 100 nmol/L Endothelin (counts/min)			Stimulated with 15 $\mu\text{g/mL}$ LDL (counts/min)		
	InsP_1 †	InsP_2 ‡	InsP_3 §	InsP_1 †	InsP_2 ‡	InsP_3 §
0.0	421	142	62	432	129	66
0.3	398	215	115	415	138	60
1.0	626	725	158	423	142	68
3.0	701	572	148	442	125	57
6.0	1626	1219	179	415	132	62
10	2507	1544	246	410	119	64
15	2924	1446	218	390	131	58
20	3031	788	138	423	125	62

* Distribution of SD was less than 12% of the mean ($n = 3$).

† Inositol 1-phosphate; ‡ Inositol 1,4-bisphosphate; § Inositol 1,4,5-trisphosphate.

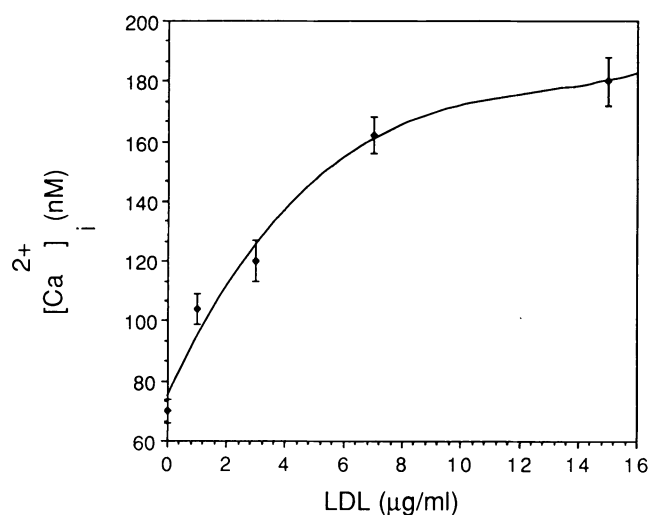


FIGURE 1. Concentration-dependent effect of 1, 3, 7, and 15 $\mu\text{g}/\text{mL}$ LDL on $[\text{Ca}^{2+}]_i$. As $[\text{Ca}^{2+}]_i$ was maximal within 15 sec, $[\text{Ca}^{2+}]_i$ obtained at 15 sec stimulations were plotted and given as mean \pm SD, $n = 4$.

LDL Changes pH_i in Normal and Receptor Negative Fibroblasts Figure 3 illustrates the effect of LDL on pH_i of receptor negative fibroblasts in HEPES buffer. Addition of 7 $\mu\text{g}/\text{mL}$ LDL exerted in both cell types a biphasic effect on pH_i (basal value = 7.04 ± 0.03 , $n = 10$). Initially, within 2 min a rapid acidification of 0.06 ± 0.02 ($n = 5$) pH units occurred, followed by a subsequent prolonged alkalinization of 0.12 ± 0.04 pH units. Similar results were obtained with normal fibroblasts.

DISCUSSION

Recently, low concentrations of LDL (1 to 30 $\mu\text{g}/\text{mL}$) induced elevation of $[\text{Ca}^{2+}]_i$ in VSMC were reported.⁷ This elevation was partly due to mobilization of calcium from intracellular stores. In addition, LDL caused pH_i shifts in VSMC, probably via activation of Na^+/H^+ exchange. Furthermore, a mitogenic effect of LDL was also demonstrated.^{16,17} It is recognized that such intracellular changes are mainly triggered by vasoactive agonists, eg, AII or endothelin, or by the receptor-dependent PtIns turnover signal system.¹⁸ These PtIns turnover-activated intracellular changes are putative signals for cell constriction and cell proliferation and consequently may play an important role in the development of hypertension.¹⁹ Since LDL showed similar intracellular changes, we examined whether the classical LDL receptor is involved in the elevation of $[\text{Ca}^{2+}]_i$ and pH_i . Surprisingly, our results indicate that these LDL induced intracellular changes are independent of the classical LDL receptor and do not occur via stimulation of the PtIns turnover signal system. These findings are supported by the following observations.

In contrast to endothelin, which caused up to a 5-fold

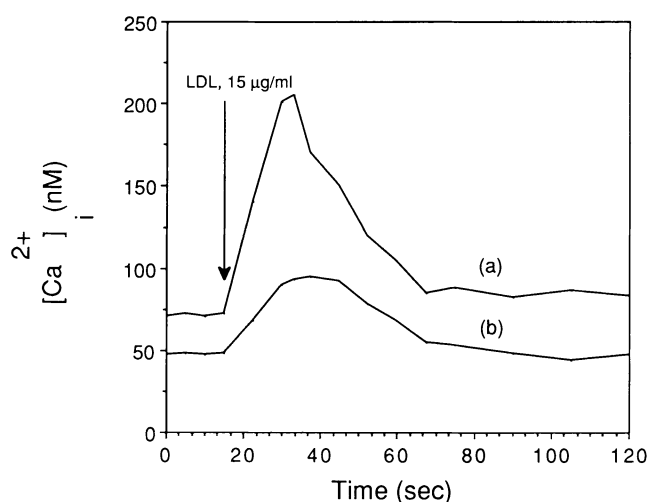


FIGURE 2. Effect of 15 $\mu\text{g}/\text{mL}$ LDL on $[\text{Ca}^{2+}]_i$ in cultured human fibroblasts in the presence and absence of extracellular Ca^{2+} . LDL (15 $\mu\text{g}/\text{mL}$) was applied to fura-2-loaded LDL receptor negative fibroblasts in HEPES buffer, containing 1 mmol/L CaCl_2 (a) and in Ca^{2+} -free HEPES-buffer, containing 1 mmol/L EGTA (b). Changes in fluorescence were monitored for 3 min. After subtraction of autofluorescence, changes in 340/380 nm excitation wavelength ratio by the emission wavelength of 505 nm were converted into corresponding levels of $[\text{Ca}^{2+}]_i$.

and 25-fold increase of InsP_3 and InsP_2 formation, respectively, LDL was not able to stimulate the formation of the PtIns-turnover metabolites InsP_1 , InsP_2 , and InsP_3 . Our results are thus in contrast with recent reports^{20,21} showing that low concentrations of LDL induced an net increase of InsP_1 , InsP_2 , and InsP_3 in VSMC within 10 min. Compared with the strong effects of classical vasoactive agonists on inositol phosphate formation, these increases appear to be of less importance for triggering cellular responses. The differences

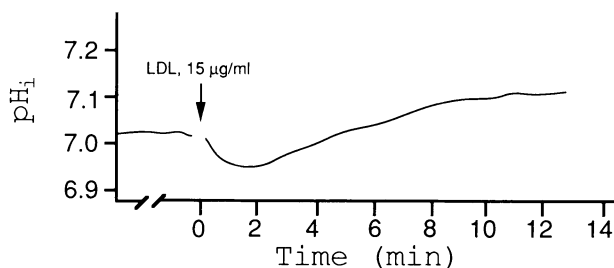


FIGURE 3. Effect of LDL on pH_i in cultured human fibroblasts. LDL (15 $\mu\text{g}/\text{mL}$) was applied to BCECF-loaded LDL receptor negative and normal human fibroblasts. Changes in fluorescence were monitored for 20 min in HEPES-buffer. After calibration of the fluorescence signal by permeabilizing the cells with 30 $\mu\text{mol}/\text{L}$ digitonin, changes in 492/438 nm excitation wavelength ratio by the emission wavelength 525 nm were converted into corresponding levels of pH_i .

in the results of our study with those of others may reflect VSMC differences, depending on the isolation procedure, strain and age of the animal, cultivation conditions, and number of passages. The much higher sensitivity of the HPLC method in separating inositol phosphates,¹³ compared to the customary method¹² using Dowex columns, would be another possible explanation for differing reports on the effect of LDL on PtlIns turnover.

Preincubation of the VSMC cultures with monoclonal antibodies (IgGC₇)⁸ against specific surface LDL receptor could not abolish or attenuate the intracellular effects of LDL.

Apolipoprotein B-100, a constituent of LDL, is a hydrophobic polypeptide with a molecular mass of 513 kDa. It facilitates the endocytosis of LDL after binding on the classical LDL receptor.² As shown in our experiments, apolipoprotein B-100 cannot stimulate the formation of inositol polyphosphates. Additionally, preincubation of the VSMC cultures with apolipoprotein B-100 could not affect the LDL induced elevation of $[Ca^{2+}]_i$.

Similar LDL-induced changes of $[Ca^{2+}]_i$ and pH_i in normal and receptor negative fibroblasts were observed, indicating that the observed LDL induced intracellular changes are independent of the specific LDL receptor. The lack of LDL to activate PtlIns turnover in both cell types is consistent with the former findings.

Since LDL induced an approximately 2-fold increase of the $^{45}Ca^{2+}$ influx compared with that in unstimulated cells,¹⁷ it can be assumed that LDL has some relation to Ca^{2+} channels. Opening of nonspecific cation channels, which induce depolarization of the membrane and thus trigger the activity of Ca^{2+} channels, leads to an elevation of $[Ca^{2+}]_i$. Low density lipoprotein induced mobilization of Ca^{2+} from intracellular stores may be brought about by an up to now unknown mediator. One example of such a mediator is GTP, which in pancreatic cells mobilizes Ca^{2+} from intracellular stores.²² According to another report,²³ indicating that an influx of extracellular Ca^{2+} mobilized the release of Ca^{2+} from the sarcoplasmic reticulum in VSMC, the possibility exists that the LDL induced release of Ca^{2+} from intracellular stores may be triggered by a similar mechanism. Our results do not exclude the existence of another LDL receptor in VSMC or fibroblasts. A nonclassical LDL receptor, the "scavenger receptor" has been described in monocyte/macrophages and endothelial cells. This receptor recognizes and takes up modified LDL at a rate many times higher than native LDL.²⁴

From our findings two principal questions arise: Is the isolated LDL native or modified? Although isolation of LDL was performed in the presence of the antioxidants BHT or ascorbic acid, a minimized oxidation of LDL cannot be excluded. Are there any artifactual LDL adherent factors causing the observed intracellular

changes? After LDL precipitation by 70% ethanol no activity in the 70% ethanol supernatant was found (unpublished observation) indicating that the observed intracellular changes were not induced by adherent factors with a small molecular mass. However, nondialyzable factors cannot be excluded and it is conceivable that these putative factors have stimulatory effects only in association with the LDL particle.

Although the real concentrations of LDL in the extracellular fluid are unknown, experiments on the LDL clearance in several tissues indicate that LDL can occur in the extracellular space.^{25,26} The relatively low in vitro concentrations of LDL triggering the aforementioned cellular events probably correspond to in vivo concentrations of LDL in extravascular fluids. These LDL concentrations are much lower than those in plasma due to the diffusion barrier offered by vascular endothelium. In addition, there are fluctuations in LDL concentrations due to dietary,²⁷ physiological,²⁸ and pharmacological manipulation.²⁹ In addition, binding and uptake of LDL have been observed following incubation of cultured rat VSMC with 10 $\mu g/mL$ of homologous LDL.^{30,31}

In conclusion, our findings suggest that LDL, apart from its physiological function as a cholesterol transport molecule, can also contribute to the pathogenesis of cardiovascular diseases by elevating $[Ca^{2+}]_i$ and pH_i . Consequently, in humans with hypercholesterolemia, LDL-cholesterol might modify vascular reactivity without involving the specific LDL receptor.

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